

**STUDIES OF A NOVEL DRUG DESIGNED TO PREVENT TUMOR
INVASION AND METASTASIS**

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**STUDIES FOR A NOVEL DRUG DESIGNED TO PREVENT
TUMOR INVASION AND METASTASIS**

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF FIGURES	vii
LIST OF SYMBOLS AND ABBREVIATIONS	viii
SUMMARY	ix
<u>CHAPTER</u>	
1 INTRODUCTION	1
1.1 Background	1
1.2 Specific Aims	4
1.3 Literature Review	4
2 PRELIMINARY <i>IN VITRO</i> INVASION ASSAYS	8
2.1 Introduction	8
2.2 Methods	8
2.3 Results	10
2.4 Discussion	11
3 <i>IN VITRO</i> STUDIES OF IMIPRAMINE BLUE	13
3.1 Introduction	13
3.2 Methods	13
3.3 Results	15
3.4 Discussion	21
4 MOVING FORWARD <i>IN VIVO</i>	22
4.1 Overall Discussion of <i>In Vitro</i> Testing	22
4.2 <i>In Vivo</i> Model	23

4.3 Conclusion	24
REFERENCES	25
BIOGRAPHY	27

LIST OF FIGURES

	Page
Figure 1.1: Imipramine Blue shows inhibition of invasion in glioblastoma <i>in vitro</i> .	2
Figure 1.2: Imipramine Blue displays <i>in vivo</i> invasion inhibition in glioblastoma.	3
Figure 1.3: Invasion Inhibition in other cancer models.	4
Figure 2.1: <i>In vitro</i> invasion assay.	10
Figure 2.2: Testing of Imipramine Blue on MatLyLu <i>in vitro</i> .	11
Figure 3.1: Effects of IB below 2 μ M on MatLyLu and RT2 on invasion.	16
Figure 3.2: Cytotoxicity of IB on MatLyLu at doses below 2 μ M.	16
Figure 3.3: Cytotoxicity of IB on RT2 at doses below 2 μ M.	17
Figure 3.4: Invasion assay for MatLyLu and RT2 with IB doses below 10 μ M.	18
Figure 3.5: 48 hour reading of MatLyLu and RT2 invasion assay.	19
Figure 3.6: Day 7 reading of MatLyLu and RT2 invasion assay.	20
Figure 4.1: <i>In vivo</i> tumor model for non-fluorescent MatLyLu cells.	24

LIST OF SYMBOLS AND ABBREVIATIONS

IB	Imipramine Blue
MatLyLu	Prostate cancer cell line
RT2	Rat Glioblastoma cell line

SUMMARY

Anti-invasive therapeutics provide a means to improve the prognosis of invasive cancer patients. Recently, one such compound have been identified for its ability to halt the invasion of glioblastoma. This compound, known as Imipramine Blue (IB), successfully reduced cancer cell invasion *in vitro* and *in vivo*, and displayed the potential to reduce invasion in other invasive cancer models as well.¹ Therefore, it was determined that the effects of IB should be studied in depth on other invasive cancer models, and a metastatic prostate cancer cell line, MatLyLu, was chosen for the study. Prostate cancer, like glioblastoma, has a poor prognosis due to its extremely invasive nature. Specific aims for this project were identified, and a literature review was conducted to evaluate other anti-invasive compounds that are currently under investigation.

The primary goal of this project was to determine the ability of IB to halt invasion of the MatLyLu cell line at non-cytotoxic levels. To do this, an *in vitro* invasion assay was performed on MatLyLu. Preliminary results showed that MatLyLu experiences a dose dependent decrease in invasion, but for this experiment, IB was determined to be cytotoxic. Thus, the invasion assay procedure was then optimized to eliminate cytotoxicity, and any possible human error. The invasive glioblastoma cell line, which has been shown to respond to IB treatment, was also incorporated as a positive control. Another test was performed, and results showed that IB was no longer cytotoxic. However, a dose dependent decrease in invasion was no longer seen for either cell line. Therefore, the invasion assay procedure was optimized further, and the experiments were performed again. Results from these experiments showed that the RT2 cells do experience a dose dependent decrease in invasion at non-cytotoxic levels. However, the

MatLyLu cell line still did not show a dose dependent decrease in invasion. There is still additional experimental optimization that can be performed, so results thus far have not provided enough evidence to determine the anti-invasive effects of IB on the MatLyLu cell line.

The second aim of this project is to develop an *in vivo* tumor model for MatLyLu. This cell line is known to metastasize, “mat,” in the lymph nodes, “ly,” and the lungs, “lu.” A metastatic model was developed in Copenhagen rats, and the model develops metastases in the lungs 18 days after tumor cell implantation. For final *in vivo* experiments this model must be tested with Luciferase expressing MatLyLu cells in order to evaluate metastasis non-invasively. Therefore, to complete this project, optimization of the *in vitro* and *in vivo* models must be finalized. However, once the last few experiments are completed, this project will be ready to move forward with *in vivo* testing of the anti-metastatic capability of IB.

CHAPTER 1

INTRODUCTION

1.1 Background

Invasive cancers are the most deadly class of cancers because they are not effectively eliminated by current treatment procedures. This is because resection, radiation, and chemotherapy do not typically affect the ability of surviving cancer cells to invade and metastasize elsewhere. Current researchers have proposed that adding an anti-invasive component to cancer treatment will increase the effectiveness of the treatment.^{1-5,7,13} Recently, there has been a shift toward developing novel drugs that target specific cell signaling pathways that induce invasion. These drugs would allow for more personalized cancer treatment because treatment would be adjusted for each patient. In these cases, the goal would be to increase the effectiveness of treatment while decreasing the adverse side effects.

Current research is geared towards evaluating new compounds and compound derivatives that specifically inhibit the mechanics of cell invasion and metastasis. Using this approach, Munson et al. discovered the novel compound, Imipramine Blue (IB), and evaluated its anti invasive properties using an invasive glioma model.¹ It was determined that IB showed dose dependent invasion inhibition *in vitro*. *In vivo* studies were then performed, and it was determined that IB decreased the invasive properties of the glioma cells resulting in more compact tumors which could be treated more effectively with cytotoxic chemotherapeutics. Although the mechanism of IB is still unknown, it is believed to inhibit cell invasion. IB belongs to a class of compounds known as triphenylmethanes, and is expected to be a universally good candidate for invasion

reduction. Therefore, Munson et al. also performed *in vitro* invasion assays on a metastatic prostate cancer model, DU145, and two metastatic breast cancer models, ZR-75 and MDA-MB435 to test this theory. The results from these studies showed a 50% decrease in invasion. Therefore, it was determined that the effects of IB should be studied on other invasive cancer models to determine its full effect on cancer invasion and metastasis.

The current project evaluates IB for its ability to halt tumor metastasis on a metastatic prostate cancer cell line. Initial studies investigated the use of IB to prevent metastasis of the cell line, Dunning R 3327 MatLyLu. *In vitro* invasion assays and cytotoxicity assays were performed to determine the ability of IB to prevent the invasion potential of this cell line. These tests were also performed with the glioblastoma line, RT2, tested by Munson et al. Results of the RT2 tests were compared to the MatLyLu tests to determine whether or not IB is capable of inhibiting cell invasion below cytotoxic levels in this cell line.

1.2 Specific Aims

There are two primary aims for this project, with one secondary aim. The primary aims of this project are to optimize the dose of IB on the metastatic prostate cell line, MatLyLu, used for invasion prevention *in vitro*, and determine if this dose is below cytotoxic levels. The goal is to determine the smallest dose of IB that causes at least a 50% reduction in invasion, so that this dose can later be used in *in vivo* studies. The secondary aim of this project is to develop an *in vivo* tumor model with MatLyLu for future *in vivo* studies of IB.

1.3 Literature Review

1.3.1 Introduction

In the past five years, there has been a significant increase in compounds that can potentially halt tumor invasion. The traditional strategy of evaluating such compounds is to determine their inhibition of matrix metalloproteinases (MMPs), and thereby their limiting the invasiveness of the cancer cells. Some new anti-invasive agents, such as epigallocatechin 3-gallate aim to induce apoptosis, or programmed cell death.² However, these compounds are non-specific and much of the research on those compounds is focused on cytotoxicity. Research is now geared towards evaluating compounds that inhibit specific cell receptors or signaling pathways in order to kill cancer cells.

Triphenylmethanes are a class of compounds that have recently been discovered to inhibit tumor angiogenesis.⁷ The purpose of this section is to review major advances in anti-invasive compound research, including the evaluation of MMP inhibitors Batimastat and Marimastat, epigallocatechin 3-gallat, and the novel triphenylmethane compounds.

1.3.2 Inhibiting matrix metalloproteinases

MMPs are enzymes that are responsible for the turnover of the extracellular matrix, and they are regulated by gene expression and secretion of proenzymes. In pancreatic cancer, tumors secrete excessive amounts of MMP-2 and MMP-9 and decreased amounts of their inhibitors TIMP-1 and TIMP-2, which cause increased turnover of the extracellular matrix. Elevated levels of MMP-2 are associated with cell invasion and subsequent metastasis, and are found in most forms of invasive cancer.⁴ Therefore, inhibiting MMPs would limit the invasive ability of cancer cells.^{3,4} Batimastat and marimastat, both MMP-2 inhibitors, were considered to be “forerunners” for the

treatment of invasive tumors.⁴ Pre-clinical trials using a glioma model were performed on rodents using marimastat and batimastat. Although the marimastat trials yielded no results due to the speedy metabolism of the compound by the rodents, batimastat yielded promising anti-invasive results. Because of the similarity between batimastat and marimastat, the results of batimastat indicated that marimastat should be successful in upcoming clinical trials.³ However, by Phase II of clinical trials marimastat was unsuccessful in enhancing positive clinical outcomes in glioblastoma cancer.⁵ Following these trials, MMP inhibitors have still been heavily pursued, but more and more evidence suggests that the assumptions about what role MMP plays in invasion must be re-evaluated.⁶

1.3.3 Apoptosis Stimulation and Induction

Non-traditional anti-invasion research evaluates compounds that competitively inhibit the genetic receptors that start apoptosis, and compounds that can stimulate apoptosis. One such compound, evaluated for its anti-invasive properties showed promising results. This compound, epigallocatechin 3-gallate (EGCG), is the major component in green tea and is currently being investigated as a cancer preventative therapeutic. EGCG binds to the GRP78 receptor, which then sensitizes the cancer cell to other chemotherapeutic agents that stimulate apoptosis. It has been shown that EGCG has sensitized a variety of different cancer models to many agents that stimulate apoptosis, such as 5-fluorouracil, taxol, vinblastine, gemcitabine, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) *in vitro*, and doxorubicin, paclitaxel, or interferon in mouse tumor models *in vivo*.² EGCG has also been shown to increase the survival of mice with glioblastoma when used in conjunction with temozolomide.

However, low concentrations of EGCG in the absence of an apoptosis stimulant showed an increase in cancer cell proliferation.²

1.3.4 Novel Compounds for Invasion Inhibition

Inhibition of tumor angiogenesis as an approach for cancer treatment was first evaluated by Judah Folkman.¹¹ Angiogenesis is the proliferation of new blood vessels, which is triggered by nitric oxide and reactive oxygen species. These free radicals are found in elevated levels inside of tumors, and regulate pathways for initiation of tumor invasion. Triphenylmethanes are a group of compounds that act to inhibit NADPH Oxidase (Nox), which is the enzyme that regulates the invasion initiation pathways.¹³ Triphenylmethanes were originally identified in 1992 by Cooney et al. as “a novel chemopreventive agent”.¹³ The invasion initiation pathways include Src kinase, p38/MAPK, and activation of NFkB, with subsequent secretion of MMPs, which is very important for invasion inhibition, as indicated by previous research.⁷ This makes triphenylmethanes and their derivatives good candidates for anti-invasion research. One such compound, IB, is a triphenylmethane derivative that has shown invasion reduction in glioblastoma.¹

1.3.5 Conclusion

The discovery of IB reveals a new possibility for anti-invasion research. Although the mechanism of IB's invasion inhibition is yet to be fully elucidated, research performed by Munson et al. suggests that it may affect a signaling pathway that is present in all invasive cancers. Moving forward with this discovery, the anti-invasive properties of IB should be evaluated on other metastatic cell lines. It should also be tested in conjunction

with radiation therapy to determine if it can negate the increased invasiveness of cancer cells caused by radiation exposure.

CHAPTER 2

PRELIMINARY *IN VITRO* INVASION ASSAYS

2.1 Introduction

Preliminary studies evaluated the use of IB in preventing the metastasis of prostate cancer model, Dunning R 3327 MatLyLu, below cytotoxic levels. This tumor model has an extremely high metastatic rate, greater than 75% in all inoculated animals, as reported by Isaacs et al.¹⁵ The glioblastoma model, RT2, used by Munson et al. was also tested and used as a positive control for the experiments. Both MatLyLu and RT2 cells were maintained in culture. Invasion assays were performed with varying doses of IB over a time period of 24 hours.

2.2 Methods

2.2.1 Cell Culture

RT2 cells were sustained in T-75 flasks with Dulbecco's Modified Eagle's Medium supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin, 1% Non-Essential Amino Acids, and 10% Fetal Bovine Serum (FBS). MatLyLu cells were sustained in T-75 flasks with Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 1% Penicillin/Streptomycin, 1% Dexamethisone, and 10% Fetal Bovine Serum (FBS). Cell lines were split once every 7 days, and media was changed once every 3-4 days.

2.2.2 *In Vitro* Invasion Assays

In vitro invasion assays were performed on MatLyLu cells to determine the optimal dose of IB for *in vivo* testing. RT2 cells were used as a positive control. Cells were treated with varied doses of IB, and 24 hours later fluorescent images were taken using

DAPI nuclear stain. To begin testing, cell culture inserts with 0.8 μm pores were placed in 24-well plates (Micell). The membranes of inserts were coated with 80 μL of 1X basement membrane extract (BME) (Cultrex) in 72% sterile, deionized water, and 8% coating buffer. Inserts were incubated for at least two hours to allow the BME to gel, and then excess is removed by aspiration. Cells were suspended in serum-free media at a concentration of 2.5×10^5 cells/mL. Serum-containing media is loaded on the outside of the inserts, which were incubated for 30 minutes to allow cell adhesion. Cells were then treated with varying doses of IB. Doses tested were 10 μM , 1 μM , 0.1 μM and 0 μM ; each dose had a sample size of $n=3$. The invasion assay procedure is depicted in Fig. 2.1. Inserts were then incubated overnight, allowing invading cells to migrate through the basement membrane, where they attach to the bottom of the well insert. Non-invading cells remain on the top of the inserts.

After 24 hours cells are fixed with 4% paraformaldehyde and stained with DAPI nuclear stain for 5 minutes. After staining, the top of each insert is wiped with a cotton swab to remove any cells on top of the insert. Five images are then taken from each well, and the cells in each image were counted. The cell counts from the five images are then averaged for each well, and standard deviation was calculated.

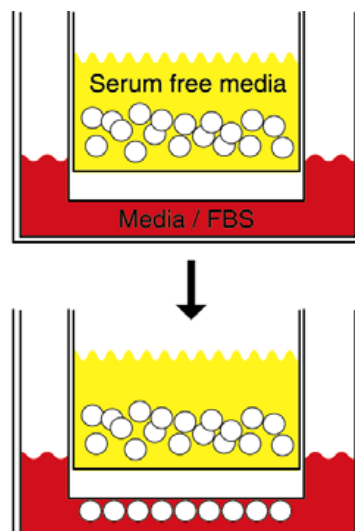


Figure 2.1: *In vitro* invasion assay. Cells were suspended inside a well-insert and treated with IB. Overnight, invading cells migrate through the basement membrane of the insert and adhere to the bottom. Cells that do not invade remain on top. Cells on the bottom were stained with DAPI and fluorescently imaged.

2.3 Results

Invasion assays were performed on MatLyLu and RT2 cells. The MatLyLu invasion assay appeared to show a dose-dependent decrease in invasion (Fig. 2.2). Cytotoxicity was also tested, and it was determined that all doses of IB tested were cytotoxic. Thus, preliminary invasion assays have yielded no conclusive results.

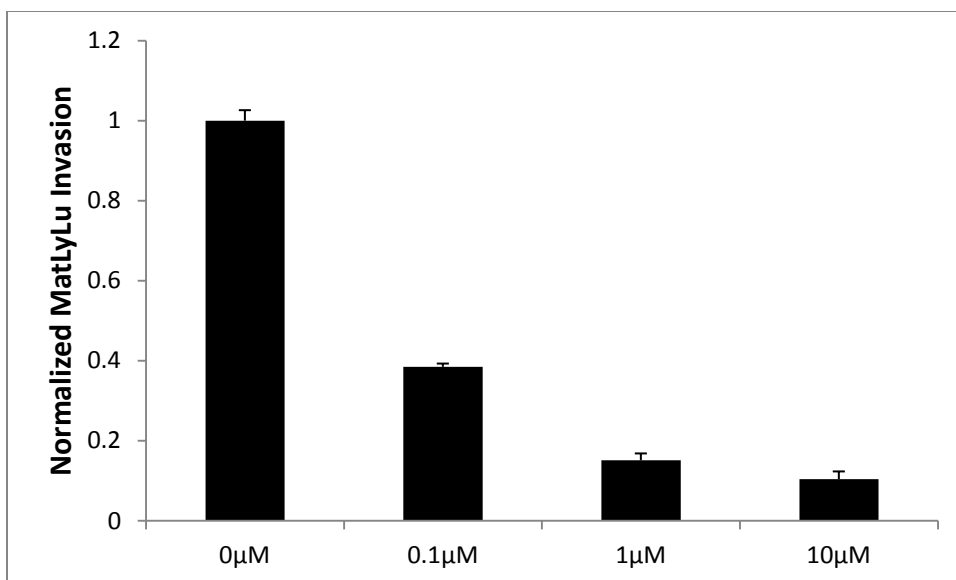


Figure 2.2: Imipramine Blue was tested on MatLyLu *in vitro*. An *in vitro* invasion assay was performed on MatLyLu to test the ability of IB to prevent invasion. This study yielded no conclusive results because all doses of IB were determined to be cytotoxic.

2.4 Discussion

Although there was a dose dependent decrease in invasion, all doses of IB tested were cytotoxic. Previously, IB showed a dose dependent decrease in invasion at these doses, but they were non-cytotoxic. Therefore, it cannot be determined if the decrease of invasion occurred because IB inhibits the invasion signaling pathway, or because IB killed more cells before they had time to invade. The cytotoxicity was caused by using a 10 μ M stock solution of IB in ethanol. It was determined that the ethanol may have degraded the IB from its original structure. Thus, for all future experiments, IB in ethanol solutions must be made fresh before treating cells. This should result in all IB treatments below 10 μ M to be non-cytotoxic, as seen previously.

The inconclusive results of the preliminary invasion assays were likely caused by the cytotoxic preparation of IB, however, the results could also be influenced by human

error. First, wiping the top of the inserts with a cotton swab does not ensure that all of the cells counted are actually on the bottom of the inserts. The DAPI stain used is also able to go through the basement membranes of the inserts and stain the cells on the top.

Additionally, the manual cell counting method is subject to human error if there were overlapping cells, or if there were cells out of focus. Finally, the results of cytotoxicity assay could be caused over-seeding the cells. If the cell density is too high, cell death that is not caused by IB will occur due to cell crowding. To improve the accuracy of future experiments, these possible sources for error should be eliminated.

CHAPTER 3

***IN VITRO* STUDIES OF IMIPRAMINE BLUE**

1.1 Introduction

In order to eliminate the human error of the *in vitro* experiments, some changes were made to the invasion assay and cytotoxicity protocols. First, fluoroblocking well inserts were used in all subsequent invasion assays. These inserts eliminate the need to wipe the top of the wells with a cotton swab because they do not let the fluorescent light through to the top of the insert. Additionally, GFP fluorescing cells were used, and invading cells were counted with a fluorescent plate reader. Using a fluorescent plate reader eliminates the human error of manual cell counting, and also eliminates the need for fixing with paraformaldehyde and DAPI staining. Eliminating the DAPI staining procedure provides the possibility to read the invasion assay at multiple time points. Finally, the cell seeding density for the cytotoxicity study was decreased to ensure that cell death due to over-confluence did not occur.

3.2 Methods

3.2.1 Invasion Assays

Invasion assays were performed on MatLyLu cells in conjunction with RT2 cells to determine the optimal dose of IB for future *in vivo* testing. Cells were treated with varied doses of IB, 0 μ M, 0.2 μ M, 1 μ M, 2 μ M, and with a sample size of n=3. Assays are performed in fluoroblocking cell culture transwells with 0.8 μ m pores that are set in 24 well plates. The tops of the transwells were coated with 80 μ L of 0.1X basement

membrane extract in 1X coating buffer (Cultrex). The BME was changed from 1X to 0.1X in order to allow more cells to invade through the transwells. Inserts were incubated for 24 hours, and then excess liquid is removed by aspiration. Cells were suspended in serum-free media and seeded at a density of 1.5×10^6 cells per well. Cell seeding density was reduced to 1×10^5 cells/well in later experiments. Serum-containing media is loaded on the outside of the inserts to promote cell migration. Transwells were then incubated for 30 minutes to allow cell adhesion before IB was added. IB was prepared by dissolving in ethanol (10 mM), then further diluted with serum free media before adding to each well for a final concentration ranging from 0-10 μ M IB. Final ethanol content in each well was 0.5%. After treating with IB, inserts were incubated overnight to allow invading cells to migrate through the BME gel. Fluorescence was read with 485/520 excitation/emission from the bottom of each well to identify GFP expressing cells that invaded through the gel. Invasion was evaluated at 24, 48 and 72 hours.

3.2.2 Cytotoxicity Assays

Cytotoxicity assays were performed on MatLyLu cells in conjunction with RT2 cells to determine if the tested doses of IB were below cytotoxic levels. Cells were treated with varied doses of IB (0 μ M, 0.2 μ M, 1 μ M, 2 μ M) and with a sample size of 3. A single well in a 24 well plate had 1×10^4 cells/well. After 2.5 hours, the drug is added to the wells, and the plate was incubated at 37°C overnight. After 24 hours, the drug is removed and a 10% cell-counting kit 8 (CCK-8) solution in serum containing media is added to each well. The CCK-8 indicates NADH production from the dehydrogenase enzyme by changing the fluorescence of the cell solution. Viable cells will turn the CCK-8 solution

yellow. As the cells die, less NADH will be produced, due to less enzyme activity, and the solution will remain red. Cell viability is measured by fluorescence at 24, 48, and 72 hours with an absorbance of 450 nm.

3.3 Results

Invasion assays were performed on MatLyLu and RT2 cells. The doses of IB tested on MatLyLu were 0 μ M, 0.2 μ M, 1 μ M, and 2 μ M. The doses tested on RT2 were 0 μ M, and 2 μ M. At a seeding density of 1.5e6 cells/well, neither cell line showed a dose-dependent decrease in invasion (Fig. 3.1). However, cytotoxicity was tested on both cell lines at the same doses of IB, and none of the tested doses showed cytotoxicity (Fig. 3.2 and 3.3). Thus, the invasion assays of Fig. 3.1 have yielded no conclusive results.

However, another invasion assay study was performed on both cell lines after reducing cell seeding density to 1e5 cells/well. Doses tested on the MatLyLu line were 0 μ M, 0.2 μ M, 1 μ M, 2 μ M, and 10 μ M. Doses tested on the RT2 cell line were 0 μ M, 2 μ M, and 10 μ M. Results from this experiment show a dose-dependent decrease in invasion in the RT2 cell line, but not in the MatLyLu cell line, seen in Fig. 3.4. This assay was read again at 48 hours (Fig. 3.5), and then again after 7 days (Fig. 3.6), but the results were the same.

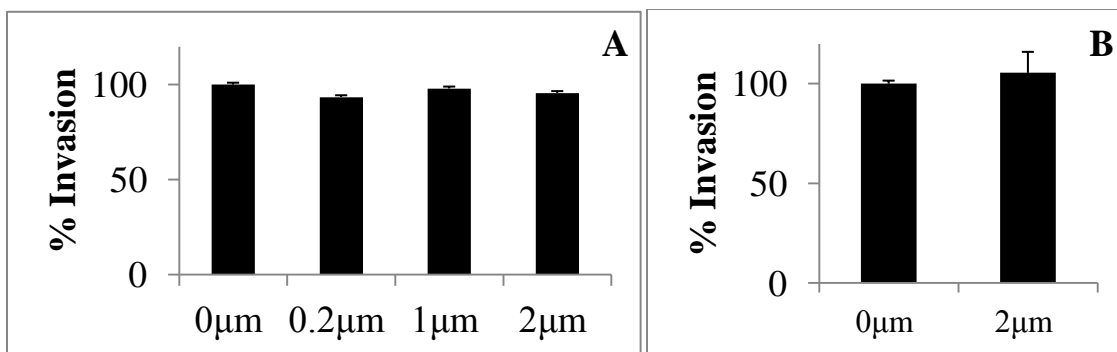


Figure 3.1: Effects of IB below 2 μ M on MatLyLu (A) and RT2 (B) on invasion. *In vitro* invasion assays were performed on MatLyLu (A) and RT2 (B) cell lines. Doses tested on MatLyLu were 0.2 μ M, 1 μ M, and 2 μ M. The dose tested on RT2, the positive control, was 2 μ M. The negative control for both cell lines was 0 μ M IB. A dose dependent decrease of invasion was not seen in either cell line at seeding density, 1.5e6 cells/well.

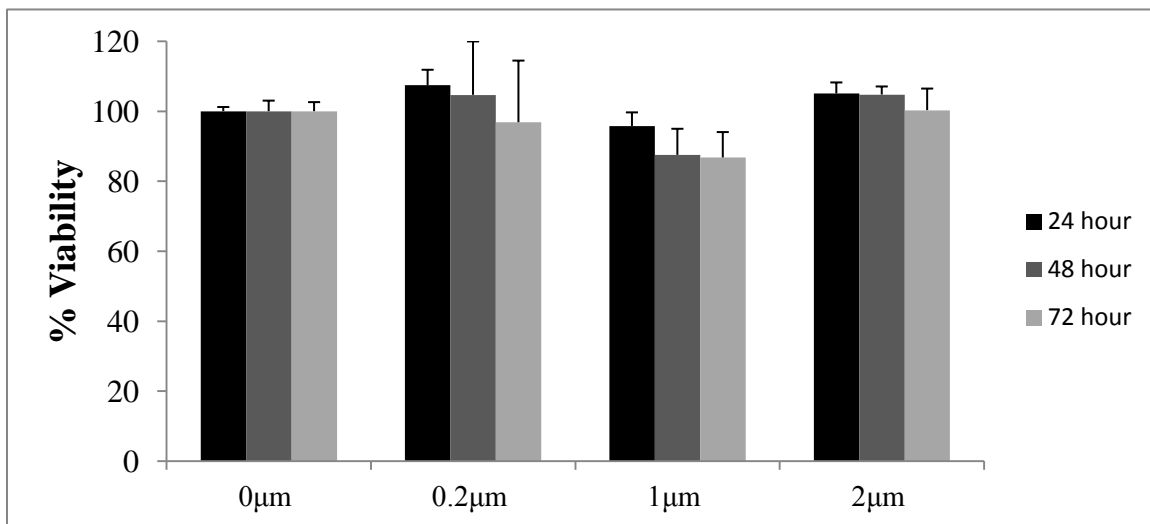


Figure 3.2: Cytotoxicity of IB on MatLyLu at doses below 2 μ M. An *in vitro* cytotoxicity assay was performed on the MatLyLu cell line. Doses tested were 0 μ M, 0.2 μ M, 1 μ M, and 2 μ M. Results show that all doses tested were non-cytotoxic.

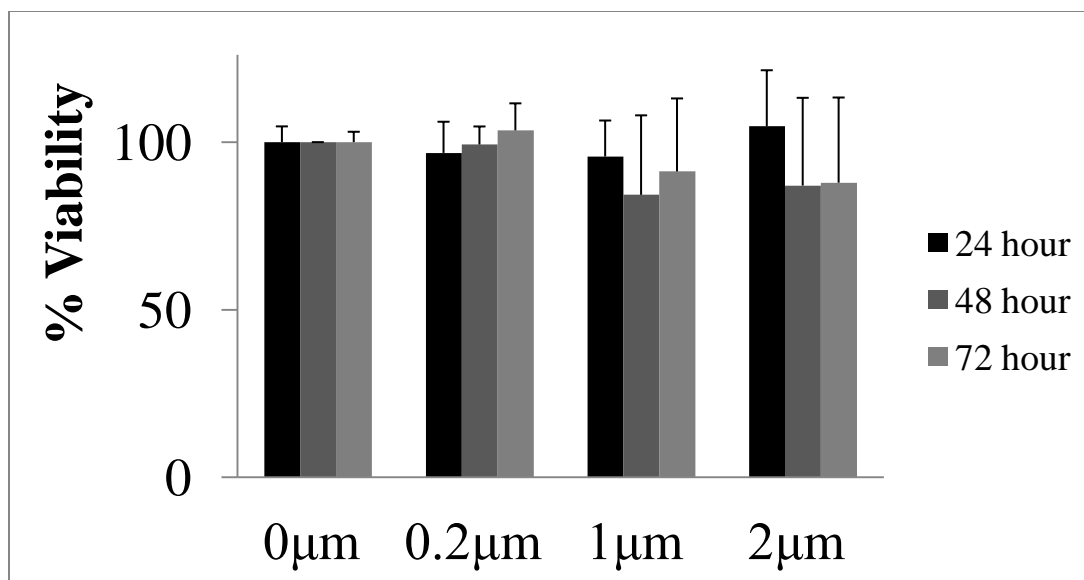


Figure 3.3: Cytotoxicity of IB on RT2 at doses below 2 μM. An *in vitro* cytotoxicity assay was performed on the MatLyLu cell line. Concentration of IB tested were 0 μM, 0.2 μM, 1 μM, and 2 μM. Results show that all doses tested were non-cytotoxic.

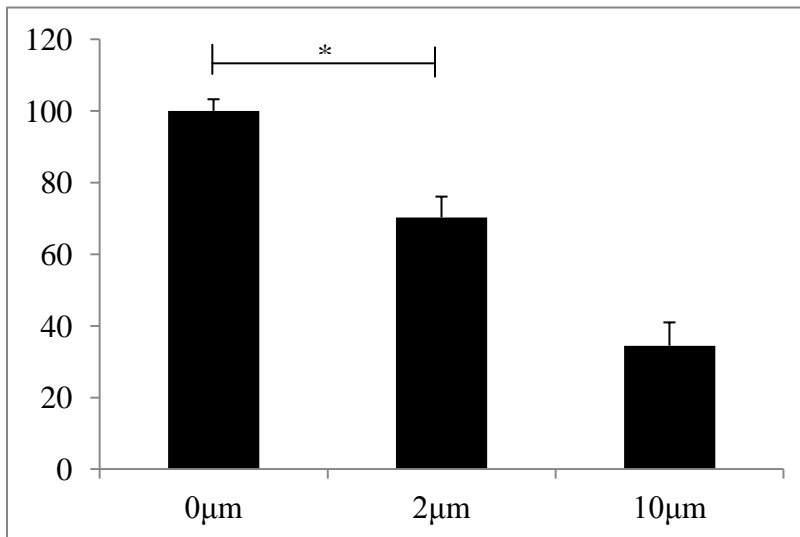
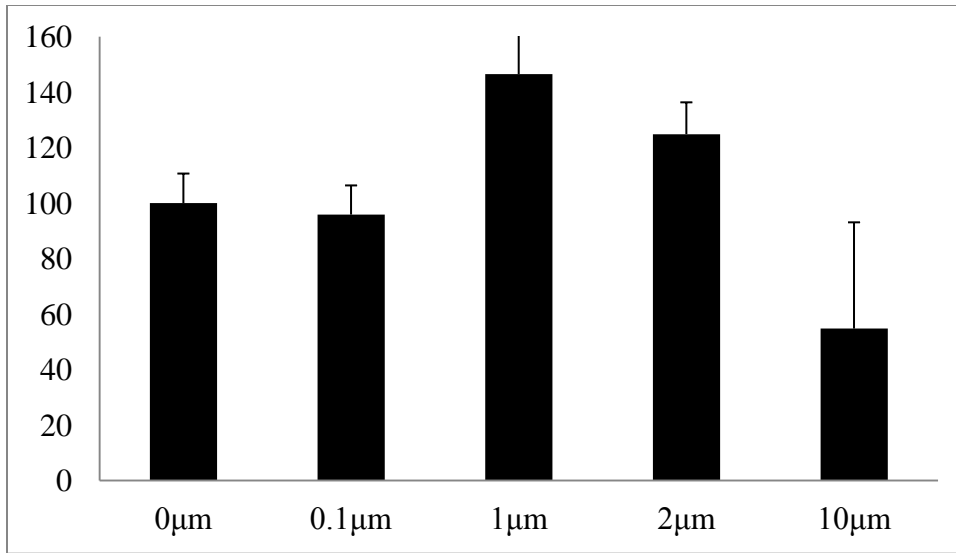


Figure 3.4: Invasion assay of MatLyLu and RT2 with IB doses below 10 µM. *In vitro* invasion assays were performed on MatLyLu and RT2 cell lines. Doses tested on MatLyLu were 0 µM, 0.2 µM, 1µM, 2 µM, and 10 µM. Doses tested on RT2 were 0 µM, 2 µM, and 10 µM. A dose dependent decrease of invasion was seen in RT2, but not in MatLyLu after 24 hour exposure. An unpaired, two-tailed t-test was performed to test for significance of RT2 invasion between 0 µM and 2 µM IB. A statistically significant decrease in invasion was determined and is indicated by an asterisk (p-value <0.005*).

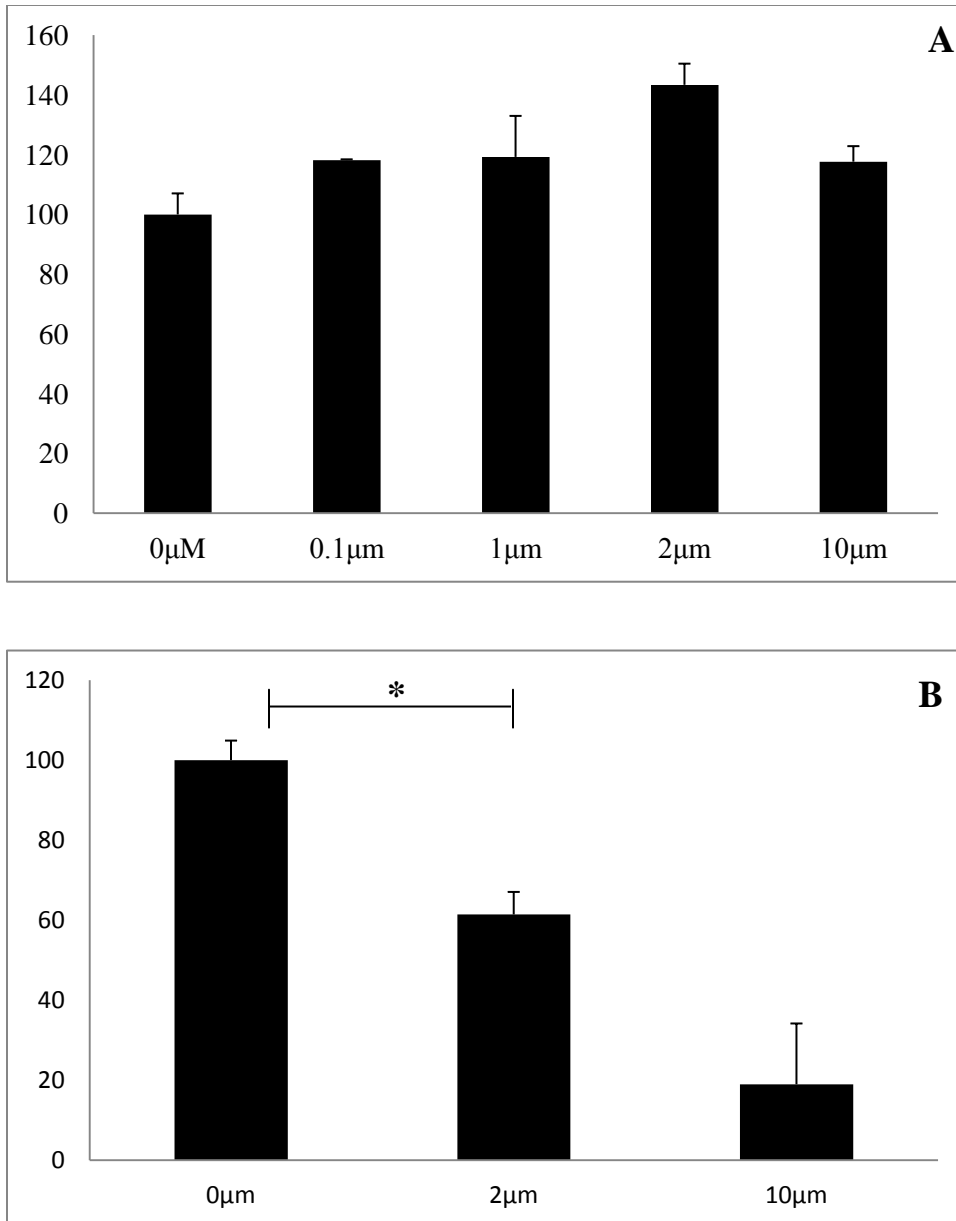


Figure 3.5: 48 hour reading of MatLyLu (A) and RT2 (B) invasion assay. *In vitro* invasion assays were incubated for an additional 48 hours, and fluorescence was measured. A dose dependent decrease of invasion was still seen in RT2, but not in MatLyLu. An unpaired, two-tailed t-test was performed to test for significance of RT2 invasion between 0uM and 2uM IB. Results were found to be statistically significant and is indicated with an asterisk (p-value <0.005*).

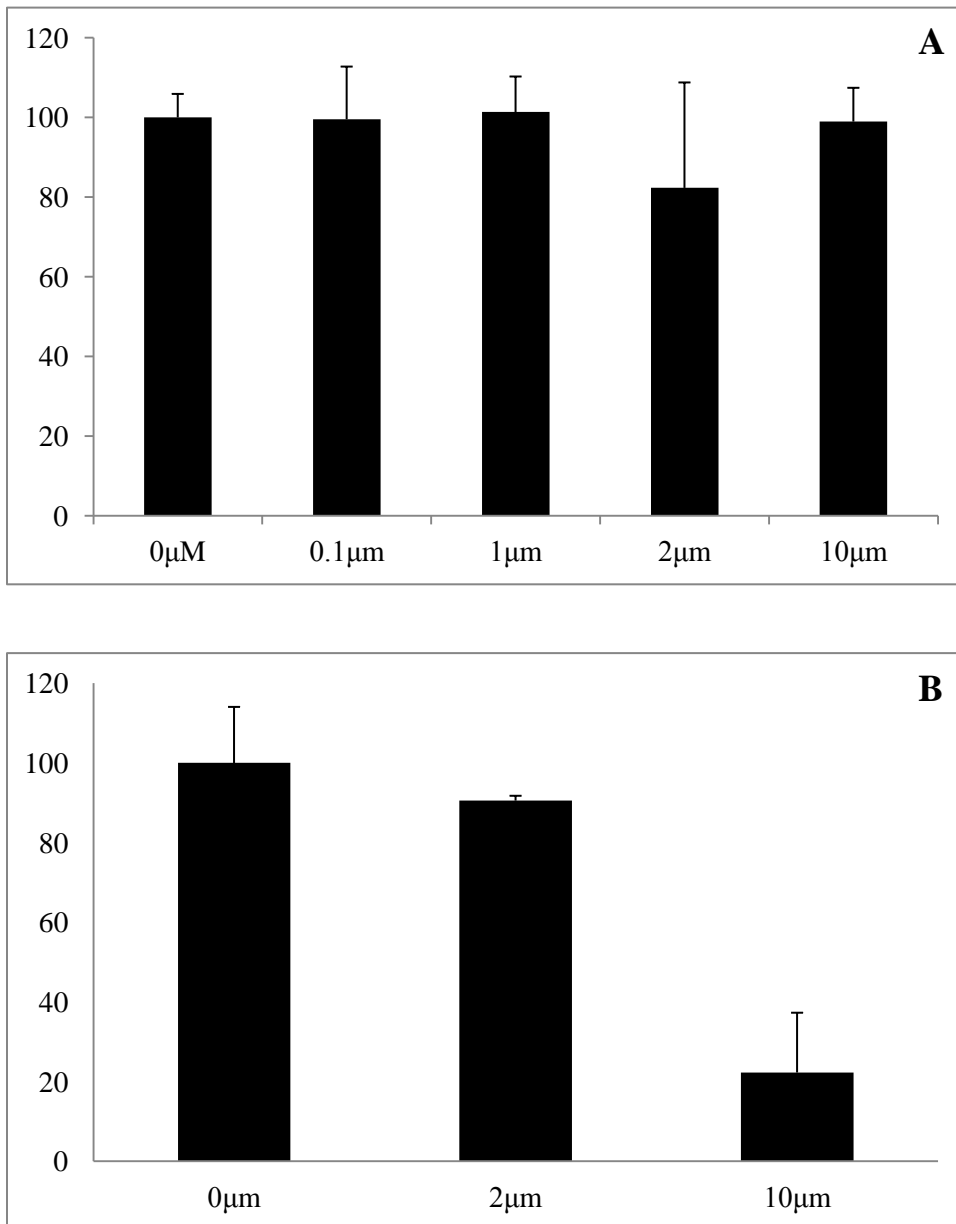


Figure 3.6. Day 7 reading of MatLyLu (A) and RT2 (B) invasion assay. *In vitro* invasion assays were monitored for a week, and on day 7, fluorescence was measured. A dose dependent decrease of invasion was still seen in RT2, but no significant change was seen in MatLyLu invasion. An unpaired, two-tailed t-test was performed to test for

significance of RT2 invasion between 0uM and 2uM IB. No statistical significance was determined (p-value >0.05*).

3.4 Discussion

Results from Figs. 3.1-3.3 indicate that although IB is no longer cytotoxic and that there is no dose dependent decrease in invasion. However, this result is likely caused by the high cell seeding density of the invasion assay. For this assay, the cell seeding density was increased significantly, from 5e5 cells/well to 1.5e6 cells/well. An extremely high cell seeding density was chosen to ensure the plate reader would be able to read the fluorescence of the cells. However, once it was determined that using the plate reader was a viable method for evaluating the number of invaded cells, the seeding density was decreased for the final experiment.

Therefore, a final invasion assay with a cell seeding density of 1e5 cells/well was performed. The results showed that the RT2 cells experienced a dose dependent decrease in invasion, as expected, and at least the 2 μ M dose was known to be non-cytotoxic. This result indicates that the new method for testing invasion is a viable one. However, a dose dependent decrease in invasion was not observed for the MatLyLu cells, even after 7 days. On day 7 the invasion assay was observed under a fluorescent microscope and images revealed that none of the MatLyLu cells, IB treated or not, experienced significant invasion. Thus, it was determined that the reason a dose dependent decrease of invasion was not seen was because the cells did not invade under any condition.

CHAPTER 4

MOVING FORWARD *IN VIVO*

4.1 Overall Discussion of *In Vitro* Testing

The future direction of cancer treatment includes the addition of anti-invasive agents to supplement current cancer treatment. Recently there have been significant advances in the development and evaluation of anti-invasive compounds. Specifically, Munson et al. tested IB on a highly invasive glioblastoma model. Their research showed that IB significantly decreased invasion *in vitro* at non-cytotoxic levels. Because IB is thought to act by disrupting a signaling pathway cells use for invasion, it is believed to be a universally good anti-invasive therapeutic.

However, results thus far have not provided enough evidence to determine whether or not IB halts the invasion of the MatLyLu cell line. In the most recent invasion assay experiment, none of the MatLyLu cells invaded, including the cells not treated with IB. Conversely, the glioblastoma cell line did experience a dose dependent decrease in invasion. This supports the results of Munson et al. and indicates that the method for performing the invasion assay is adequate.

A final invasion assay experiment should be optimized to promote MatLyLu invasion. At a density of 1.5×10^6 cells/well, invasion was observed, but no reduction was observed. At 1×10^5 cells/well, invasion was not observed. Thus, the most likely cause of the MatLyLu's lack of invasion is the cell seeding density. Although this is the optimal cell seeding density for the RT2 cells, RT2 cells are known to be more invasive than MatLyLu cells. A higher cell seeding density may be needed to further promote the

MatLyLu cells to invade. To complete this section of the project, a seeding density optimization experiment should be performed on the MatLyLu cell line. Once determined, a final invasion assay experiment should be performed to evaluate IB's ability to halt the invasion of MatLyLu.

4.2 *In Vivo* Model

To complete the secondary aim of this project, an *in vivo* tumor model for MatLyLu was developed. Since MatLyLu is known to invade in the lymph nodes and the lungs, the lungs of tumor bearing animals were explanted and examined for metastases. Primary tumors are surgically resected 10 days after implantation to promote MatLyLu metastasis. MatLyLu cells were passaged *in vivo* by inoculating cells into the left flank of Copenhagen rats. Once the tumors reached 3 cm in diameter, they were explanted. The tumor tissue was subjected to mechanical dissociation in serum free media to obtain a suspension of tumor cells. These cells were then inoculated subcutaneously into the left flank of a new rat. Cells were passaged in this manner until they were ready to be utilized for *in vivo* testing. For the metastatic model, approximately 2.5×10^6 *in vivo* passaged MatLyLu cells were inoculated into the left flank of rats. Tumors were surgically resected once they reached 3 cm in diameter. The animals were then allowed to recover, and were euthanized 8 days later.

Lungs from healthy animals did not show signs of metastasis, and lungs from cancerous animals were covered with metastases, as seen in Figure 4.1. This model was developed using genetically unaltered MatLyLu cells. However, in future experiments this model should be tested with luciferase expressing cells. Luciferase expressing cells allows the opportunity to examine metastasis non-invasively, which could allow animal

survival studies to be performed in the future. Animals with luciferase expressing tumors can be treated with luciferin, and the cells can be tracked through bioluminescent imaging to the metastatic sites. Luciferin is then excreted from the body, without causing harm to the animal.

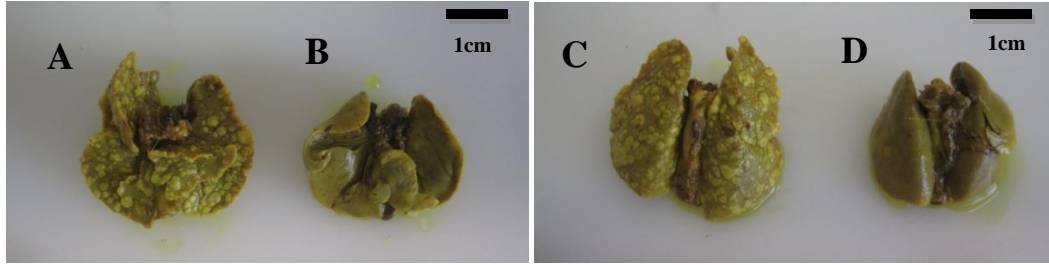


Figure 4.1: *In vivo* tumor model for non-fluorescent MatLyLu cells. Dorsal (A,B) and ventral (C,D) views of lungs explanted from rats are present in the lung from the cancer-bearing animal (A,C) but not in the lung from the healthy animal (B,D). Scale bar is 1cm.

4.3 Conclusion

In conclusion, more experiments need to be done for both the *in vitro* and *in vivo* models before the effects of IB can be tested *in vivo* on MatLyLu. The lowest effective dose of IB on MatLyLu is yet to be determined, although the experimental method for testing this has been finalized. Additionally, the *in vivo* tumor model should be tested with luciferase expressing cells so metastasis can be tracked non-invasively. Once both of these tests are finalized, this project will be ready to move forward to test IB *in vivo*.

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VITA

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